IN THE SPECIFICATION:

Applicants submit the following amendments to the specification pursuant to 37 C.F.R. § 1.121:

Kindly ***substitute*** the following two contiguous paragraphs in place of the corresponding two contiguous paragraphs beginning on page 8, line 17 and extending through page 9, line 13:

--Figure 3 shows a flow diagram of the inventive process in several, but not all, alternative embodiments for PCR product analysis. Variations in detection methodology, such as the use of dual probe fluorescence energy transfer ("FRET") technology ~~(Lightcycler®)~~ (LIGHTCYCLER®) or fluorescent primers FRET technology ~~(Sunrise® technology)~~ (SUNRISE® technology) are not shown in this Figure. Specifically, the inventive process begins with a mixed sample of genomic DNA that is converted in a sodium bisulfite reaction to a mixed pool of methylation-dependent sequence differences according to standard procedures (the bisulfite process converts unmethylated cytosine residues to uracil). Fluorescence-based PCR is then performed either in an "unbiased" PCR reaction with primers that do not overlap known CpG methylation sites (left arm of Figure 3), or in a "biased" reaction with PCR primers that overlap known CpG dinucleotides (right arm of Figure 3). Sequence discrimination can occur either at the level of the amplification process (C and D) or at the level of the fluorescence detection process (B), or both (D). A quantitative test for methylation patterns in the genomic DNA sample is shown on the left arm (B), wherein sequence discrimination occurs at the level of probe hybridization. In this version, the PCR reaction provides for unbiased amplification in the presence of a fluorescent probe that overlaps a particular putative methylation site. An unbiased control for the amount of input DNA is provided by a reaction in which neither the primers, nor the probe overlie any CpG dinucleotides (A). Alternatively, as shown in the right arm of Figure 3, a qualitative test for genomic methylation is achieved by probing of the biased PCR pool with either control oligonucleotides that do not "cover" known methylation sites (C; a fluorescence-based version of the MSP technique), or with oligonucleotides covering potential methylation sites (D).

Figure 4 shows a flow chart overview of the inventive process employing a dual label "TaqMan®" probe in the amplification process. Briefly, double-stranded genomic DNA is treated with sodium bisulfite and subjected to one of two sets of PCR reactions using dual label TaqMan® probes; namely with either biased primers and dual label TaqMan® probe (left column), or unbiased primers and dual label TaqMan® probe (right column). The dual label TaqMan® probe is dual-labeled with a fluorescent "reporter" (labeled "R" in Figure 4) and "qencher" (labeled "O") molecules, and is designed to be specific for a relatively high GC content region so that it melts out at about 10 °C higher temperature in the PCR cycle than the forward or reverse primers. This

allows it to remain fully hybridized during the PCR annealing/extension step. As the Taq polymerase enzymatically synthesizes a new strand during PCR, it will eventually reach the annealed dual label TaqMan® probe. The Taq polymerase 5' to 3' endonuclease activity will then displace the dual label TaqMan® probe by digesting it to release the fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent system as described herein.--

Kindly ***substitute*** the following paragraph in place of the corresponding paragraph on page 10, lines10-19:

--Figure 8 illustrates a comparison of *MLH1* expression, microsatellite instability and *MLH1* promoter methylation of 25 matched-paired human colorectal samples. The upper chart shows the *MLH1* expression levels measured by quantitative, real time RT-PCR (dual label TaqMan®) in matched normal (hatched bars) and tumor (solid black bars) colorectal samples. The expression levels are displayed as a ratio between *MLH1* and *ACTB* measurements. Microsatellite instability status (MSI) is indicated by the circles located between the two charts. A black circle denotes MSI positivity, while an open circle indicates that the sample is MSI negative, as determined by analysis of the *BAT25* and *BAT26* loci. The lower chart shows the methylation status of the *MLH1* locus as determined by an inventive process. The methylation levels are represented as the ratio between the *MLH1* methylated reaction and the *MYOD1* reaction.--

Kindly ***substitute*** the following paragraph in place of the corresponding paragraph on page 10, lines 28-34:

--In contrast to previous methods for determining methylation patterns, detection of the methylated nucleic acid is relatively rapid and is based on amplification-mediated displacement of specific oligonucleotide probes. In a preferred embodiment, amplification and detection, in fact, occur simultaneously as measured by fluorescence-based real-time quantitative PCR ("RT-PCR") using specific, dual-labeled dual label TaqMan® oligonucleotide probes. The displaceable probes can be specifically designed to distinguish between methylated and unmethylated CpG sites present in the original, unmodified nucleic acid sample.--

Kindly ***substitute*** the following paragraph in place of the corresponding paragraph beginning on

page 12, line 32 and extending through page 13, line 7:

--As disclosed by MSP inventors, "[t]he only technique that can provide more direct analysis than MSP for most CpG sites within a defined region is genomic sequencing." (US Patent 5,786,146 at 5, line 15-17). The present invention provides, in fact, a method for the partial direct sequencing of modified CpG sites within a known (previously sequenced) region of genomic DNA. Thus, a series of CpG-specific dual label TaqMan® probes, each corresponding to a particular methylation site in a given amplified DNA region, are constructed. This series of probes are then utilized in parallel amplification reactions, using aliquots of a single, modified DNA sample, to simultaneously determine the complete methylation pattern present in the original unmodified sample of genomic DNA. This is accomplished in a fraction of the time and expense required for direct sequencing of the sample of genomic DNA, and are substantially more sensitive. Moreover, one embodiment of the present invention provides for a quantitative assessment of such a methylation pattern.--

Kindly ***substitute*** the following paragraph in place of the corresponding paragraph on page 15, lines 18-29:

--Preferably, as employed in the embodiment of Application D, the amplification process provides for amplifying bisulfite converted nucleic acid by means of two oligonucleotide primers in the presence of a specific oligonucleotide hybridization probe. Both the primers and probe distinguish between modified unmethylated and methylated nucleic acid. Moreover, detecting the "methylated" nucleic acid is based upon amplification-mediated probe fluorescence. In one embodiment, the fluorescence is generated by probe degradation by 5' to 3' exonuclease activity of the polymerase enzyme. In another embodiment, the fluorescence is generated by fluorescence energy transfer effects between two adjacent hybridizing probes (fluorescence energy transfer ("FRET") LIGHTCYCLER® ~~Lightcycler®~~ technology) or between a hybridizing probe and a primer. In another embodiment, the fluorescence is generated by the primer itself (FRET SUNRISE® ~~Sunrise®~~ technology). Preferably, the amplification process is an enzymatic chain reaction that uses the oligonucleotide primers to produce exponential quantities of amplification product, from a target locus, relative to the number of reaction steps involved.--

Kindly ***substitute*** the following ten contiguous paragraphs in place of the corresponding ten contiguous paragraphs beginning on page 16, line 7 and extending through page 19, line 16:

--Preferably, methylation-dependent sequence differences are detected by methods based on fluorescence-based quantitative PCR (real-time quantitative PCR, Heid et al., *Genome Res.* 6:986-994, 1996; Gibson et al., *Genome Res.* 6:995-1001, 1996) (*e.g.*, dual label "TaqMan®," fluorescence energy transfer ("FRET") LIGHTCYCLER®, ~~"Lightcycler®,"~~ and FRET SUNRISE® ~~"Sunrise®"~~ technologies). For the dual label TaqMan® and FRET LIGHTCYCLER® ~~Lightcycler®~~ technologies, the sequence discrimination can occur at either or both of two steps: (1) the amplification step, or (2) the fluorescence detection step. In the case of the FRET SUNRISE® ~~"Sunrise®"~~ technology, the amplification and fluorescent steps are the same. In the case of the FRET hybridization, probes format on the FRET SUNRISE® ~~Lightcycler®~~, either or both of the FRET oligonucleotides can be used to distinguish the sequence difference. Most preferably the amplification process, as employed in all inventive embodiments herein, is that of fluorescence-based Real Time Quantitative PCR (Heid et al., *Genome Res.* 6:986-994, 1996) employing a dual-labeled fluorescent oligonucleotide probe (dual label TaqMan® PCR, using an ABI Prism 7700 Sequence Detection System, Perkin Elmer Applied Biosystems, Foster City, California).

The dual label "TaqMan®" PCR reaction uses a pair of amplification primers along with a nonextendible interrogating oligonucleotide, called a dual label TaqMan® probe, that is designed to hybridize to a GC-rich sequence located between the forward and reverse (*i.e.*, sense and anti-sense) primers. The dual label TaqMan® probe further comprises a fluorescent "reporter moiety" and a "quencher moiety" covalently bound to linker moieties (*e.g.*, phosphoramidites) attached to nucleotides of the dual label TaqMan® oligonucleotide. Examples of suitable reporter and quencher molecules are: the 5' fluorescent reporter dyes 6FAM ("FAM"; 2,7 dimethoxy-4,5-dichloro-6-carboxy-fluorescein), and TET (6-carboxy-4,7,2',7'-tetrachlorofluorescein); and the 3' quencher dye TAMRA (6-carboxytetramethylrhodamine) (Livak et al., *PCR Methods Appl.* 4:357-362, 1995; Gibson et al., *Genome Res.* 6:995-1001; and 1996; Heid et al., *Genome Res.* 6:986-994, 1996).

One process for designing appropriate dual label TaqMan® probes involves utilizing a software facilitating tool, such as "Primer Express" that can determine the variables of CpG island location within GC-rich sequences to provide for at least a 10 °C melting temperature difference (relative to the primer melting temperatures) due to either specific sequence (tighter bonding of GC, relative to AT base pairs), or to primer length.

The dual label TaqMan® probe may or may not cover known CpG methylation sites, depending on the particular inventive process used. Preferably, in the embodiment of Application D, the dual label TaqMan® probe is designed to distinguish between modified unmethylated and methylated nucleic acid by overlapping from 1 to 5 CpG sequences. As described above for the

fully unmethylated and fully methylated primer sets, dual label TaqMan® probes may be designed to be complementary to either unmodified nucleic acid, or, by appropriate base substitutions, to bisulfite-modified sequences that were either fully unmethylated or fully methylated in the original, unmodified nucleic acid sample.

Each oligonucleotide primer or probe in the dual label TaqMan® PCR reaction can span anywhere from zero to many different CpG dinucleotides that each can result in two different sequence variations following bisulfite treatment (^{m}CpG, or UpG). For instance, if an oligonucleotide spans 3 CpG dinucleotides, then the number of possible sequence variants arising in the genomic DNA is $2^3 = 8$ different sequences. If the forward and reverse primer each span 3 CpGs and the probe oligonucleotide (or both oligonucleotides together in the case of the FRET format) spans another 3, then the total number of sequence permutations becomes 8 X 8 X 8 = 512. In theory, one could design separate PCR reactions to quantitatively analyze the relative amounts of each of these 512 sequence variants. In practice, a substantial amount of qualitative methylation information can be derived from the analysis of a much smaller number of sequence variants. Thus, in its most simple form, the inventive process can be performed by designing reactions for the fully methylated and the fully unmethylated variants that represent the most extreme sequence variants in a hypothetical example (see Figure 3, Application D). The ratio between these two reactions, or alternatively the ratio between the methylated reaction and a control reaction (Figure 3, Application A), would provide a measure for the level of DNA methylation at this locus. A more detailed overview of the qualitative version is shown in Figure 4.

Detection of methylation in the embodiment of Application D, as in other embodiments herein, is based on amplification-mediated displacement of the probe. In theory, the process of probe displacement might be designed to leave the probe intact, or to result in probe digestion. Preferably, as used herein, displacement of the probe occurs by digestion of the probe during amplification. During the extension phase of the PCR cycle, the fluorescent hybridization probe is cleaved by the 5' to 3' nucleolytic activity of the DNA polymerase. On cleavage of the probe, the reporter moiety emission is no longer transferred efficiently to the quenching moiety, resulting in an increase of the reporter moiety fluorescent-emission spectrum at 518 nm. The fluorescent intensity of the quenching moiety (*e.g.*, TAMRA), changes very little over the course of the PCR amplification. Several factors my influence the efficiency of dual label TaqMan® PCR reactions including: magnesium and salt concentrations; reaction conditions (time and temperature); primer sequences; and PCR target size (*i.e.*, amplicon size) and composition. Optimization of these factors to produce the optimum fluorescence intensity for a given genomic locus is obvious to one skilled in the art of PCR, and preferred conditions are further illustrated in the "Examples" herein. The amplicon may range in size from 50 to 8,000 base pairs, or larger, but may be smaller.

Typically, the amplicon is from 100 to 1000 base pairs, and preferably is from 100 to 500 base pairs. Preferably, the reactions are monitored in real time by performing PCR amplification using 96-well optical trays and caps, and using a sequence detector (ABI Prism) to allow measurement of the fluorescent spectra of all 96 wells of the thermal cycler continuously during the PCR amplification. Preferably, process D is run in combination with the process A (Figure 3) to provide controls for the amount of input nucleic acid, and to normalize data from tray to tray.

Application C. The inventive process can be modified to avoid sequence discrimination at the PCR product detection level. Thus, in an additional qualitative process embodiment (Figure 3, Application C), just the primers are designed to cover CpG dinucleotides, and sequence discrimination occurs solely at the level of amplification. Preferably, the probe used in this embodiment is still a dual label TaqMan® probe, but is designed so as not to overlap any CpG sequences present in the original, unmodified nucleic acid. The embodiment of Application C represents a high-throughput, fluorescence-based real-time version of MSP technology, wherein a substantial improvement has been attained by reducing the time required for detection of methylated CpG sequences. Preferably, the reactions are monitored in real time by performing PCR amplification using 96-well optical trays and caps, and using a sequence detector (ABI Prism) to allow measurement of the fluorescent spectra of all 96 wells of the thermal cylcer continuously during the PCR amplification. Preferably, process C is run in combination with process A to provide controls for the amount of input nucleic acid, and to normalize data from tray to tray.

Application B. The inventive process can be also be modified to avoid sequence discrimination at the PCR amplification level (Figure 3, A and B). In a quantitative process embodiment (Figure 3, Application B), just the probe is designed to cover CpG dinucleotides, and sequence discrimination occurs solely at the level of probe hybridization. Preferably, dual label TaqMan® probes are used. In this version, sequence variants resulting from the bisulfite conversion step are amplified with equal efficiency; as long as there is no inherent amplification bias (Warnecke et al., *Nucleic Acids Res.* 25:4422-4426, 1997). Design of separate probes for each of the different sequence variants associated with a particular methylation pattern (*e.g.*, 2^3=8 probes in the case of 3 CpGs) would allow a quantitative determination of the relative prevalence of each sequence permutation in the mixed pool of PCR products. Preferably, the reactions are monitored in real time by performing PCR amplification using 96-well optical trays and caps, and using a sequence detector (ABI Prism) to allow measurement of the fluorescent spectra of all 96 wells of the thermal cylcer continuously during the PCR amplification. Preferably, process B is run in combination with process A to provide controls for the amount of input nucleic acid, and to normalize data from tray to tray.

Application A. Process A (Figure 3) does not, in itself, provide for methylated-CpG

sequence discrimination at either the amplification or detection levels, but supports and validates the other three applications by providing control reactions for the amount of input DNA, and to normalize data from tray to tray. Thus, if neither the primers, nor the probe overlie any CpG dinucleotides, then the reaction represents unbiased amplification and measurement of amplification using fluorescent-based quantitative real-time PCR serves as a control for the amount of input DNA (Figure 3, Application A). Preferably, process A not only lacks CpG dinucleotides in the primers and probe(s), but also does not contain any CpGs within the amplicon at all to avoid any differential effects of the bisulfite treatment on the amplification process. Preferably, the amplicon for process A is a region of DNA that is not frequently subject to copy number alterations, such as gene amplification or deletion.

Results obtained with the qualitative version of the technology are described in the examples below. Dozens of human tumor samples have been analyzed using this technology with excellent results. High-throughput using a dual label TaqMan® machine allowed performance of 1100 analyses in three days with one dual label TaqMan® machine.--

Kindly ***substitute*** the following paragraph in place of the corresponding paragraph on page 21, lines 21-33:

--In a dual label TaqMan® protocol, the 5' to 3' nuclease activity of Taq DNA polymerase cleaved the probe and released the reporter, whose fluorescence was detected by the laser detector of the ABI Prism 7700 Sequence Detection System (Perkin-Elmer, Foster City, CA). After crossing a fluorescence detection threshold, the PCR amplification resulted in a fluorescent signal proportional to the amount of PCR product generated. Initial template quantity can be derived from the cycle number at which the fluorescent signal crosses a threshold in the exponential phase of the PCR reaction. Several reference samples were included on each assay plate to verify plate-to-plate consistency. Plates were normalized to each other using these reference samples. The PCR amplification was performed using a 96-well optical tray and caps with a final reaction mixture of 25 µl consisting of 600 nM each primer, 200 nM probe, 200 µM each dATP, dCTP, dGTP, 400 µM dUTP, 5.5 mM $MgCl_2$, 1X dual label TaqMan® Buffer A containing a reference dye, and bisulfite-converted DNA or unconverted DNA at the following conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.--

Kindly ***substitute*** the following paragraph in place of the corresponding paragraph beginning on page 22, line 19 and extending through page 23, line 17:

--Five sets of PCR primers and probes, designed specifically for bisulfite converted DNA sequences, were used: (1) a set representing fully methylated and fully unmethylated DNA for the *ESR1* gene; (2) a fully methylated set for the *MLH1* gene; (3) a fully methylated and fully unmethylated set for the *APC* gene; and (4) a fully methylated and fully unmethylated set for the *CDKN2A* (p16) gene; and (5) an internal reference set for the *MYOD1* gene to control for input DNA. The methylated and unmethylated primers and corresponding probes were designed to overlap 1 to 5 potential CpG dinucleotides sites. The *MYOD1* internal reference primers and probe were designed to cover a region of the *MYOD1* gene completely devoid of any CpG dinucleotides to allow for unbiased PCR amplification of the genomic DNA, regardless of methylation status. As indicated above, parallel dual label TaqMan® PCR reactions were performed with primers specific for the bisulfite-converted methylated and/or unmethylated gene sequences and with the *MYOD1* reference primers. The primer and probe sequences are listed below. In all cases, the first primer listed is the forward PCR primer, the second is the dual label TaqMan® probe, and the third is the reverse PCR primer. *ESR1* methylated (GGCGTTCGTTTTGGGATTG [SEQ ID NO. 1], 6FAM 5'-CGATAAAACCGAACGACCCGACGA-3' TAMRA [SEQ ID NO. 2], GCCGACACGCGAACTCTAA [SEQ ID NO. 3]); *ESR1* unmethylated (ACACATATCCCACCAACACACAA [SEQ ID NO. 4], 6FAM 5'-CAACCCTACCCCAAAAACCTACAAATCCAA-3'TAMRA [SEQ ID NO. 5], AGGAGTTGGTGGAGGGTGTTT [SEQ ID NO. 6]); *MLH1* methylated (CTATCGCCGCCTCATCGT [SEQ ID NO. 7], 6FAM 5'-CGCGACGTCAAACGCCACTACG-3' TAMRA [SEQ ID NO. 8], CGTTATATATCGTTCGTAGTATTCGTGTTT [SEQ ID NO. 9]); *APC* methylated (TTATATGTCGGTTACGTGCGTTTATAT [SEQ ID NO. 10], 6FAM 5'-CCCGTCGAAAACCCGCCGATTA-3' TAMRA [SEQ ID NO. 11], GAACCAAAACGCTCCCCAT [SEQ ID NO. 12]); *APC* unmethylated (GGGTTGTGAGGGTATATTTTTGAGG [SEQ ID NO. 13], 6FAM 5'-CCCACCCAACCACACAACCTACCTAACC-3' TAMRA [SEQ ID NO. 14], CCAACCCACACTCCACAATAAA [SEQ ID NO. 15]); *CDKN2A* methylated (AACAACGTCCGCACCTCCT [SEQ ID NO. 16], 6FAM 5'-ACCCGACCCCGAACCGCG-3' TAMRA [SEQ ID NO. 17], TGGAATTTTCGGTTGATTGGTT [SEQ ID NO. 18]); *CDKN2A* unmethylated (CAACCAATCAACCAAAAATTCCAT [SEQ ID NO. 19], 6FAM 5'-CCACCACCCACTATCTACTCTCCCCCTC-3' TAMRA [SEQ ID NO. 20], GGTGGATTGTGTGTGTTTGGTG [SEQ ID NO. 21]); and *MYOD1,* (CCAACTCCAAATCCCCTCTCTAT [SEQ ID NO. 22], 6FAM 5'-TCCCTTCCTATTCCTAAATCCAACCTAAATACCTCC-3' TAMRA [SEQ ID NO. 23],

TGATTAATTTAGATTGGGTTTAGAGAAGGA [SEQ ID NO. 24]).--

Kindly ***substitute*** the following paragraph in place of the corresponding paragraph on page 26, lines 16-23:

--Figure 7 shows that the mean value for the methylated reaction was higher in the tumor compared to the normal tissue whereas the unmethylated reaction showed the opposite result. The standard errors observed for the eight independent measurements were relatively modest and were comparable to those reported for other studies utilizing dual label TaqMan® technology (Fink et al., *Nature Med.* 4:1329-1333, 1998). Some of the variability of the inventive process may have been a result of stochastic PCR amplification (PCR bias), which can occur at low template concentrations. (Warnecke et al., *Nucleic Acids Res.* 25:4422-4426,1997). In summary, these results indicate that the inventive process can yield reproducible results for complex, heterogeneous DNA samples.--

Finally, kindly ***substitute*** the following four contiguous paragraphs in place of the corresponding four contiguous paragraphs beginning on page 27, line 3 and extending through page 28, line 20:

--Using the high-throughput inventive process, as described in Example 1 Application D, 50 samples consisting of 25 matched pairs of human colorectal adenocarcinomas and normal mucosa were analyzed for the methylation status of the *MLH1* CpG island. Quantitative RT-PCR (dual label TaqMan®) analyses of the expression levels of *MLH1* normalized to *ACTB* (β-actin) was investigated. Furthermore, the microsatellite instability (MSI) status of each sample was analyzed by PCR of the *BAT25* and *BAT26* loci (Parsons et al., *Cancer Res.* 55:5548-5550, 1995). The twenty-five paired tumor and normal mucosal tissue samples were obtained from 25 patients with primary colorectal adenocarcinoma. The patients comprised 16 males and 9 females, ranging in age from 39-88 years, with a mean age of 68.8. The mucosal distance from tumor to normal specimens was between 10 and 20 cm. Approximately 2 grams of the surgically removed tissue was immediately frozen in liquid nitrogen and stored at -80 °C until RNA and DNA isolation.

Quantitative RT-PCR and Microsatellite Instability Analysis. The quantitation of mRNA levels was carried out using real-time fluorescence detection. The dual label TaqMan® reactions were performed as described above for the assay, but with the addition of 1U AmpErase uracil N-glycosylase). After RNA isolation, cDNA was prepared from each sample as previously described (Bender et al., *Cancer Res* 58:95-101, 1998). Briefly, RNA was isolated by lysing tissue in buffer

containing quanidine isothiocyanate (4M), N-lauryl sarcosine (0.5%), sodium citrate (25mM), and 2-mercaptoethanol (0.1M), followed by standard phenol-chloroform extraction, and precipitation in 50% isopropanol/50% lysis buffer. To prepare cDNA, RNA samples were reverse-transcribed using random hexamers, deoxynucleotide triphosphates, and SUPERSCRIPT II® Superscript II® reverse transcriptase (Life Technologies, Inc., Palo Alto, CA). The resulting cDNA was then amplified with primers specific for *MLH1* and *ACTB*. Contamination of the RNA samples by genomic DNA was excluded by analysis of all RNA samples without prior cDNA conversion. Relative gene expression was determined based on the threshold cycles (number of PCR cycles required for detection with a specific probe) of the *MLH1* gene and of the internal reference gene *ACTB*. The forward primer, probe and reverse primer sequences of the *ACTB* and *MLH1* genes are: *ACTB* (TGAGCGCGGCTACAGCTT [SEQ ID NO. 25], 6FAM5'-ACCACCACGGCCGAGCGG-3'TAMRA [SEQ ID NO. 26], CCTTAATGTCACACACGATT [SEQ ID NO. 27]); and *MLH1* (GTTCTCCGGGAGATGTTGCATA [SEQ ID NO. 28], 6FAM5'-CCTCAGTGGGCCTTGGCACAGC-3'TAMRA [SEQ ID NO. 29], TGGTGGTGTTGAGAAGGTATAACTTG [SEQ ID NO. 30]).

Alterations of numerous polyadenine ("pA") sequences, distributed widely throughout the genome, is a useful characteristic to define tumors with microsatellite instability (Ionov et al., *Nature* 363:558-561, 1993). Microsatellite instability (MSI) was determined by PCR and sequence analysis of the *BAT25* (25-base pair pA tract from an intron of the c-kit oncogene) and *BAT26* (26-base pair pA tract from an intron of the mismatch repair gene hMSH2) loci as previously described (Parsons et al., *Cancer Res* 55:5548-5550, 1995). Briefly, segments the BAT25 and BAT26 loci were amplified for 30 cycles using one ^{32}P-labeled primer and one unlabeled primer for each locus. Reactions were resolved on urea-formamide gels and exposed to film. The forward and reverse primers that were used for the amplification of BAT25 and BAT26 were: *BAT25* (TCGCCTCCAAGAATGTAAGT [SEQ ID NO. 31], TCTGCATTTTAACTATGGCTC [SEQ ID NO. 32]); and *BAT26* (TGACTACTTTTGACTTCAGCC [SEQ ID NO. 33], AACCATTCAACATTTTTAACCC [SEQ ID NO. 34]).

Figure 8 shows the correlation between *MLH1* gene expression, MSI status and promoter methylation of *MLH1*, as determined by the inventive process. The upper chart shows the *MLH1* expression levels measured by quantitative, real time RT-PCR (dual label TaqMan®) in matched normal (hatched bars) and tumor (solid black bars) colorectal samples. The expression levels are displayed as a ratio between *MLH1* and *ACTB* measurements. Microsatellite instability status (MSI) is indicated by the circles located between the two charts. A black circle denotes MSI positivity, while an open circle indicates that the sample is MSI negative, as determined by analysis of the *BAT25* and *BAT26* loci. The lower chart shows the methylation status of the *MLH1*

locus as determined by inventive process. The methylation levels are represented as the ratio between the *MLH1* methylated reaction and the *MYOD1* reaction.--